Comparative Study of Carboxymethyl Tamarind Seed Powder Coated Chitosan/ Montmorillonite/ Hydroxyapatite Composite Macrosphere Scaffolds

Veena Vyas

Department of Biotechnology and Medical Engineering

National Institute of Technology Rourkela

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by

Veena Vyas

(Roll No. 214BM1381)

based on the research carried out

under the supervision of

Dr. A. Thirugnanam

May 2016

Department of Biotechnology and Medical Engineering

National Institute of Technology Rourkela

Department of Biotechnology and Medical Engineering

National Institute of Technology Rourkela

Date:

Certificate of Examination

Roll Number : 214BM1381

Name : Veena Vyas

Title of Dissertation : Comparative Study of Carboxymethyl Tamarind Seed Powder Coated Chitosan/ Montmorillonite/ Hydroxyapatite Composite Macrosphere Scaffolds

We the below signed, after checking the dissertation mentioned above and the official record book (s) of the student, hereby state our approval of the dissertation submitted in partial fulfillment of the requirements of the degree of *Master of Technology* in *Biomedical Engineering* at *National Institute of Technology Rourkela*. We are satisfied with the volume, quality, correctness, and originality of the work.

Prof. A. Thirugnanam

Principal Supervisor

Department of Biotechnology and Medical Engineering

National Institute of Technology Rourkela

Prof. A. Thirugnanam
Assistant Professor

Date:

Supervisor's Certificate

This is to certify that the work presented in the dissertation entitled *Comparative Study of Carboxymethyl Tamarind Seed Powder Coated Chitosan/ Montmorillonite/ Hydroxyapatite Composite Macrosphere Scaffolds* submitted by *Veena Vyas*, Roll Number *214BM1381*, is a record of original research carried out by him under my supervision and guidance in partial fulfillment of the requirements of the degree of *Master of Technology* in *Biomedical Engineering*. Neither this dissertation nor any part of has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

Prof. A. Thirugnanam
Assistant Professor

Dedicated to my Parents

For always supporting and encouraging me

Declaration of Originality

I, ***Veena Vyas***, Roll Number ***214BM1381*** hereby declare that this dissertation entitled ***Comparitive study of Carboxymethyl Tamarind seed powder coated Chitosan/ Montmorillonite/ Hydroxyapatite composite Macrosphere Scaffolds*** represents my original work carried out as a postgraduate student of NIT Rourkela and, to the best of my knowledge, it contains no material previously published or written by another person, nor any material presented for the award of any other degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the section "Bibliography". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present dissertation.

Date:

Veena Vyas

NIT Rourkela

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Date:

NIT Rourkela

Veena Vyas

Roll Number: 214BM1381

Abstract

Over the years biopolymer based composites have been replacing synthetic polymer based composites for various biomedical applications. This is attributed to the highly biocompatible and biodegradable nature of the natural polymers. Several studies have been reported pertaining to development of chitosan (CS)/ montmorillonite (MMT) and CS/hydroxyapatite (HA) composites for application in bone tissue regeneration. In the present work a CS/MMT/HA composite was developed for the fabrication of sintered macrospheric three dimensional (3D) scaffolds. Further the composite scaffolds were coated with carboxymethyl tamarind seed powder (CTSP). The scaffold fabrication method is based on direct agglomeration of the sintered macrospheres. The described method allows fabrication of porous scaffolds with controllable shape and pore size distribution. The CS/MMT/HA composite 3D scaffolds were characterized using X-ray diffraction (XRD), attenuated total reflectance-Fourier transform infrared (ATR-FTIR) and scanning electron microscopy (SEM). The scaffolds were further evaluated for their *in vitro* bioactivity, hemocompatibility, protein adsorption, water adsorption and degradation. The mechanical properties were evaluated by analysing the compressive strength. The XRD pattern confirmed the presence of the individual components in the composite scaffolds. The ATR-FTIR studies confirms the presence of molecular interaction among the various constituents of the composite. The SEM micrographs show the pore size and the interconnectivity of the scaffolds. The CS/MMT/HA composites show enhanced *in vitro* biological properties as compared to plain chitosan as well as CS/HA and CS/MMT composites. The CS/MMT/HA composite scaffolds showed significant improvement in the compressive as compared to the other samples. The present work deals with fabrication of chitosan composite scaffolds with well controllable and predictable internal architecture and geometry that has potential application in bone tissue engineering.

Keywords*: *chitosan composite, tamarind seed powder, macrosphere, 3D scaffolds.

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. Background

The human body is made up of a number of different tissues and organs which form a complex network and have specific individual functions. The aging process of the human body brings in a lot of changes which includes loosing or damaging of tissues or their functionalities [1]. Orthopaedic injuries are the main area the main area of focus when it comes to tissue repair. The major challenge faced by orthopaedic surgeons lies in repairing the fractures caused by fractures, tumours, infections, surgery and trauma. Studies have shown that every year around 900,000 patients undergo bone replacement surgery. The grafts use for these surgeries fall under three categories: autograft, allograft and xenograft [2] . When the source of the graft tissue is the same patient it is known as an autograft. It has no immunological reactions and therefore high success rates. However it involves the complication of double surgeries. Allografts are grafts where the tissue is taken from same species. These involve high risk of tissue rejection and disease transmission. When the grafts are of animal origin these are known as xenografts. The advantage of xenografts is that these are cheap and can be found in bulk but carry a high risk of tissue rejection and bacterial as well as viral infections [3]. The properties of different types of different types of grafts which help in bone tissue engineering are given in Table 1.1.

Table 1-1. Types, sources and properties of different bone grafts

Type of Graft	Source	Properties
Autograft	Same Patient	Osteogenic Osteoconductive Osteoinductive
Allograft	Same species	Osteoinductive Osteoconductive
Xenograft	Different species (animal)	Osteoconductive

1.2. Tissue Engineering

The designing and fabrication of these grafts comes under tissue engineering. With the help of engineered tissue we can replace and restore various tissue functions by delivering the cells obtained from either of the sources to a pre-engineered customized tissue construct for implantation. Till now the classical development of two dimensional (2-D) scaffolds have been widely used as grafts [4]. However in the body the cells require a three dimensional

environment for proliferating and differentiating. Generally the cells are seeded in a 3D scaffold that outlines the geometry of the replacement tissue. The fate and function of the 3D scaffold majorly depends on the cell microenvironment where coordination among the temporal, spatial, chemical and mechanical aspects Have a significant part in the graft functions. Therefore it can concluded that the selection of materials and design is the first and most crucial step in the fabrication of scaffolds [5]. Figure 1.1 shows the graphical illustration of the tissue engineering principle.

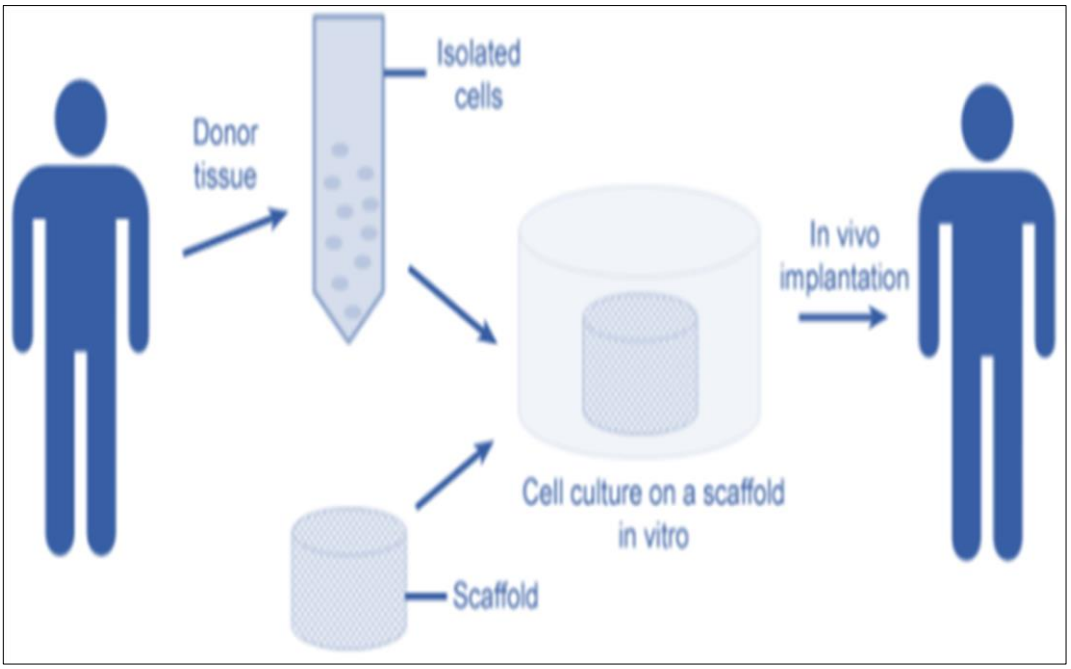


Figure 1.1. Graphical illustration of Tissue engineering principle

1.3. Materials for scaffolds

The material selection process for tissue engineering mainly depends on its biocompatibility, hemocomaptibilty, biodegradability, mechanical properties and cell viability. Other properties which come into play are the chemical and molecular structure of the material, solubility, hydrophobic/hydrophilic nature, water absorption etc. [6]. The material also affects the processing and designing of desired morphological structures. A large number of materials like polymers, metals and ceramics have been used for scaffold fabrication. The most commonly used metals are steel, cobalt alloys and titanium alloys. As these metals are non-degradable they are used for permanent applications like hip implants. Ceramics are mostly used for hard tissue engineering like bone and dental implants as they have high mechanical strength. Ceramics which commonly used for tissue engineering are alumina, zirconia, hydroxyapatite, bioglass etc. However the major disadvantage with ceramics and

metals is that these are not only non-biodegradable but also have limited processibility. Taking these factors into consideration polymeric materials have garnered significant amount of attention in the field of soft tissue engineering like muscle, nerve and skin as they can be tailored for the desired scaffold structure [7]. Polymers are of two kinds: natural and synthetic. Each type has its specific advantages and disadvantages. In recent years development of polymer composite materials for the fabrication of three dimensional scaffolds has gained a lot of interest. Both synthetic as well as natural polymers have been used for manufacturing such composites. However polymers of natural origin have been rapidly substituting the synthetic materials owing to their better biocompatibility and biodegradability [8].

1.3.1. Chitosan

Amongst all the various natural polymers available chitosan (CS) is known to have been extensively used for numerous biomedical engineering applications. Chitosan is the second most abundant polymer avalaible in nature. Chitosan is a polysaccharide derived from chitin and consisting of N-acetyl glucosamine and glucosamine linked by β (1-4) linkages where around than 60 % acetyl groups are detached via deacetylation [9]. The structure of a chitosan molecule is shown in Figure 1.2.

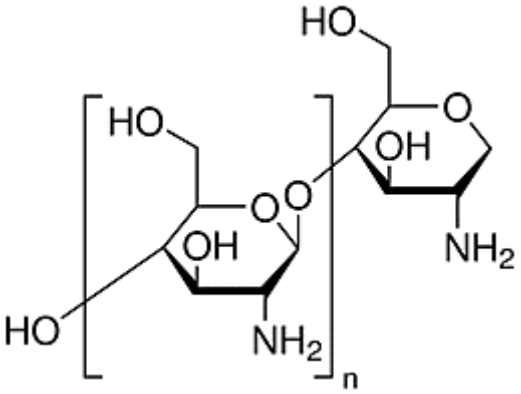


Figure 1.2. Chitosan molecule

CS has minimal foreign body interaction, excellent osteo-conductivity, suitable degradation rate and favourable wettability. It is known to be a homeostatic material with a positive influence on wound healing and bone reformation. Chitosan is structurally similar to glycosaminoglycans that are recognised to have a stimulatory effect on matrix organization. Owing to this structural similarity chitosan is also believed to have similar effects during tissue repair [10]. Several studies have been conducted on the addition of various organic

and inorganic materials to chitosan to improve its physicochemical as well as mechanical strength. Some of the materials that have been studied as an addition to chitosan to form composites hydroxyapatite (HA) and montmorillonite (MMT). The composites formed have shown not only good mechanical strength but also improved bioactivity, biodegradability, hydrophilicity and anti-bacterial properties. [11].

1.3.2. Hydroxyapatite

Hydroyapatite (HA) is widely used as one of the bioactive ceramic materials due to its close structural and compositional resemblance with that of natural bone. HA coatings are not only known to enhance the bone bonding ability of the metal surface, but also induce osteo-conductivity between the implant surface and human tissue in vivo [12]. These metal ions are known to cause serious health issues like Alzheimer's, neuropathy and osteomalacia. HA is known to stimulate osteoconductivity and can integrate into natural bone without eliciting any immune response. The studies of Kong *et al* showed that the scaffolds made from CS/HA composite were found to highly porous with enhanced bioactivity, biocompatibility and increased rate of cell proliferation compared to that of pure chitosan scaffolds [13]. HA is known to be hydrophobic material, its addition to CS reduces its water absorption capacity thereby increasing its mechanical strength. Studies have also shown that the CS/HA composite scaffolds are well tolerated by the body and are osteoconductive in nature, both of which are essential requirements in biomedical application. Pure hydroxyapatite, however, is very brittle, and its mere adhesion to metal surface add to its possible limitations under shear forces and other loads when coated on metal surfaces [14].The structure of HA molecule is shown in Figure 1.3.

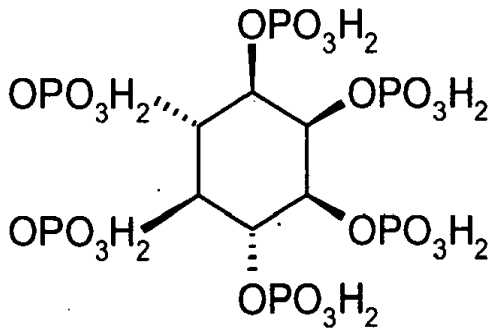


Figure 1.3. Hydroxyapatite molecule

1.3.3. Montmorillonite

Monmorillonite is a nanoclay which is a part of the smectite group of minerals. It has a layered alumina silicate structure where an octahedral sheet of aluminium hydroxide is bonded in between two tetrahedral of silica. The large surface area and high aspect ratio makes MMT a suitable filler material. The use of montmorillonite for the fabrication of PCNs was discovered by Toyotaa research group when their studies showed significant enhancement in mechanical properties of the PCNs with small montmorillonite loadings [15]. The investigations have suggested modifier usage to enhance the clay miscibility in the polymer. The functional groups and backbone present in the modifier are known to affect the d-spacing of the clay. A novel CS/HA/MMT composite with enhanced mechanical property and biocompatibility for biomedical applications was reported by Katti *et al*. Studies have sbrought to light that the addition of MMT has significantly improved the mechanical properties of polymers. The findings of Xu *et al* have shown the improved mechanical properties of the CS/MMT scaffold. There have been various other studies which show the enhanced biological properties of the CS/MMT composite including cell viability and proliferation [16].The structure of montmorillonite molecule is shown Figure 1.4.

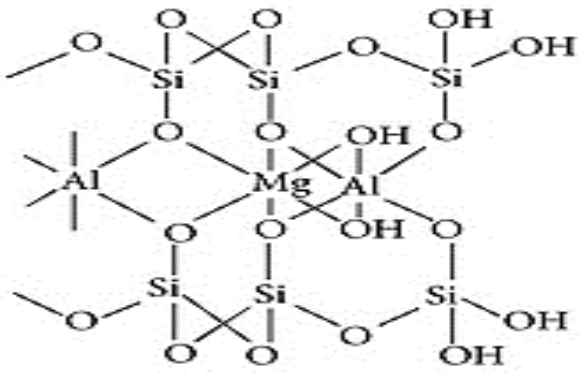


Figure 1.4. Montmorillonite molecule

1.3.4. Carboxymethyl tamarind seed powder

Tamarnd seed powder is obtained from *Tamarindus indica* and has found potential application as a pharmaceutical agent and to form gel. This biopolymer is composed of 1, 4-d-glucopyranosyl backbone which is partially substituted with 1, 6-d-xylopyranosyl side chains, a number of which are thereafter replaced with 1, 2-d-galactopyranosyl residues.

This natural form of the polymer however has a number of drawbacks. There the polymer is modified by carboxymethylation. The modification improves the polymer solubility in cold water and removes bad odour [17]. The structure of tamarind seed powder molecule is shown in Figure 1.5.

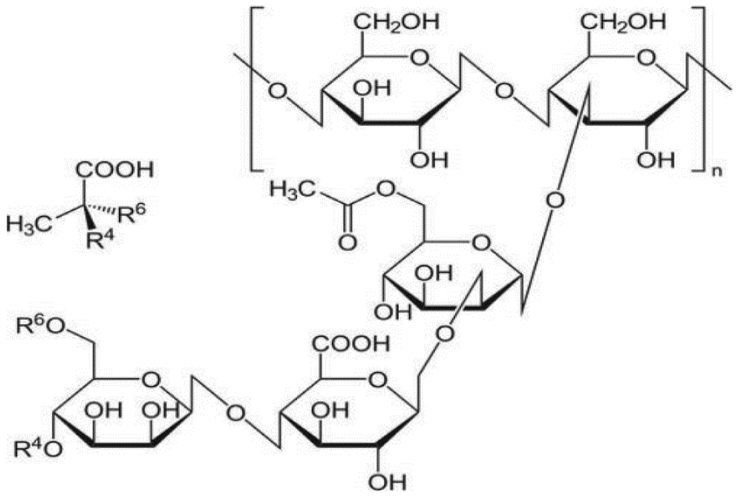


Figure 1.5. Tamarind seed powder molecule

1.4. Scaffold designing

Scaffold design and fabrication is by far the most crucial aspect of tissue engineering and regenerative research. Scaffold properties are mostly attributed to variables like pore size, microstructure, porosity, mechanical properties and surface chemistry. These above mentioned qualities are fulfilled in better way by 3-D scaffolds as compared to 2-D scaffolds. The native tissue structure of the extracellular matrix should be well mimicked by the scaffold microstructure [18]. The scaffolds should not only be sufficiently porous but should also have well interconnected pores for transport of nutrient and wastes. Besides porosity, mechanical properties of the scaffold is another major aspect of tissue designing. The scaffold should have adequate mechanical strength to resist the various stresses it is subjected to along with the physiological loads during tissue regeneration. It is preferred that the mechanical properties of the scaffold especially its Young's modulus should be similar to the corresponding tissue it is substituting, in order to avoid stress shielding. The presence of different functional groups which are present on the surface of the scaffold is also essential for the attachment of various biological molecules. The topography of the scaffold should be such that it facilitates the attachment, proliferation and differentiation of the cells. Thus

it can be concluded that scaffold design plays an important role in graft implantation [19]. Figure 1.6 shows different scaffold properties

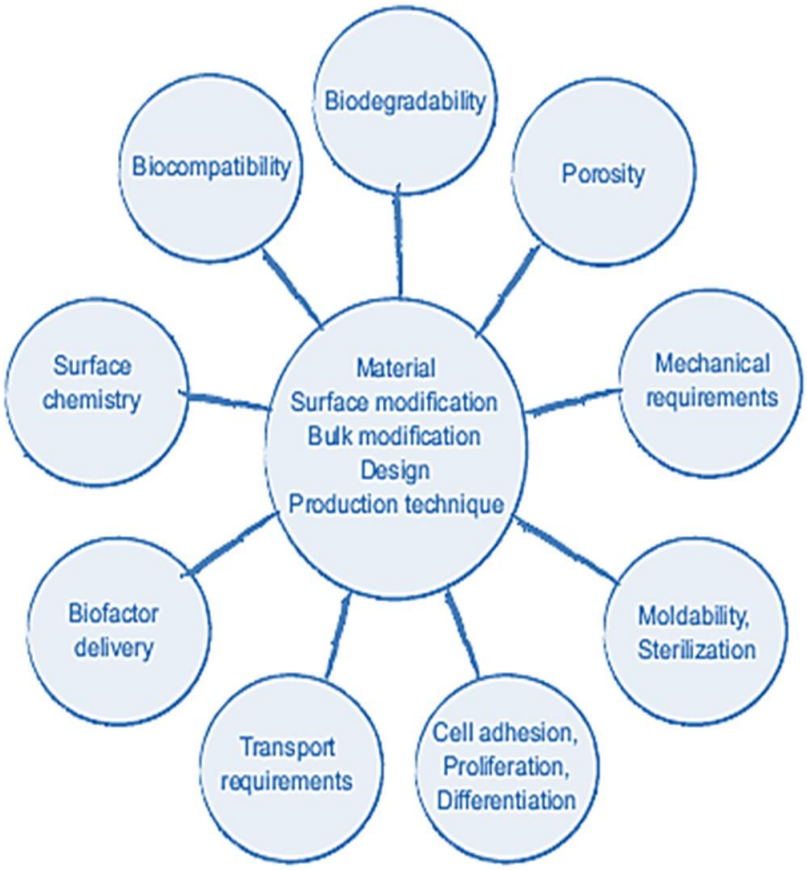


Figure 1.6. Schematic representation of scaffold properties

1.4.1. Macrospheric 3-D scaffolds

The development of three dimensional (3-D) scaffold based implants have become an important technique for bone restoration and regeneration in the field of tissue engineering. Bone scaffolds must have high porosity as well good mechanical strength. The interconnected pores are important as they are indispensable for cell ingrowth, proliferation and nutrient transport in the scaffold [20]. The mechanical properties of the graft should be similar to that of the natural bone to prevent stress shielding. However, the scaffolds with high porosity tend to have poor mechanical strength. In recent years various methods free drying, electrospinning etc. have been explored which provide polymeric scaffolds with high porosity. These scaffolds do not possess enough mechanical strength. In order to obtain bone scaffolds with both porosity and high mechanical strength we need to optimize both suitable biomaterial and the method of fabrication of scaffold. The described method allows

fabrication of scaffolds which are porous and have controllable shape and pore size distribution [21]. Figure 1.7 shows graphical illustration of the 3-D macrosphere scaffold fabrication.

Figure 1.7. Graphical illustration of 3D macrosphere scaffold fabrication

1.4.2. Curing of the 3-D macrospheric scaffolds

One of the earliest sintered macrosphere scaffolds (SMSs) were reported in 2001 by Borden *et al.* These SMSs not only aided in controlled release of drugs but also facilitated cell proliferation due to their porous structure. It was seen that compared to the traditional 3-D scaffolds, SMS significantly enhances the mechanical properties. The compressive strength of these scaffolds was found equal to that of the cancellous bone. A graphical illustration of SMS preparation is shown in Figure 1.7. Currently there are two different ways to fuse the macrospheres together for the fabrication of the scaffolds. The first method involves heating of the macropheres which are stacked in a mould, above the glass transition temperature (T_g) of for several hours. Thereafter the mould is removed to obtain the desired 3-D scaffolds. The second method involves use of a suitable solvent to bond the macrospheres together [22], [23]. Figure 1.8 shows the graphical illustration of macrosphere fusing due to sintering.

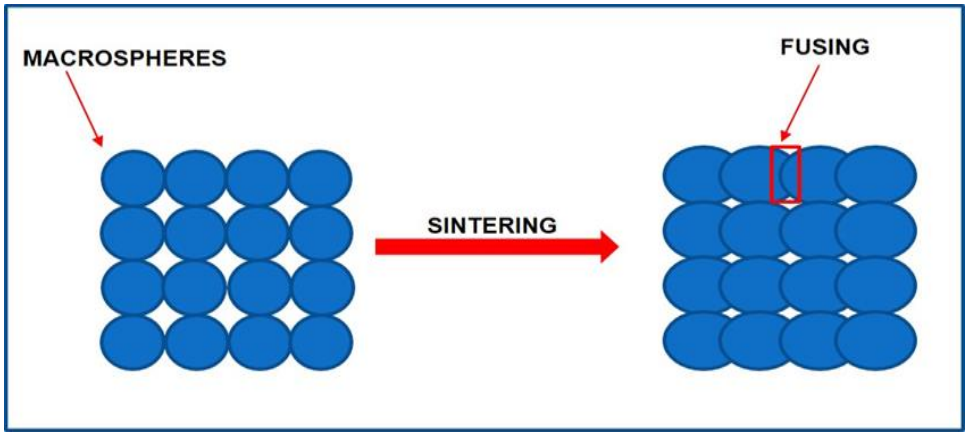


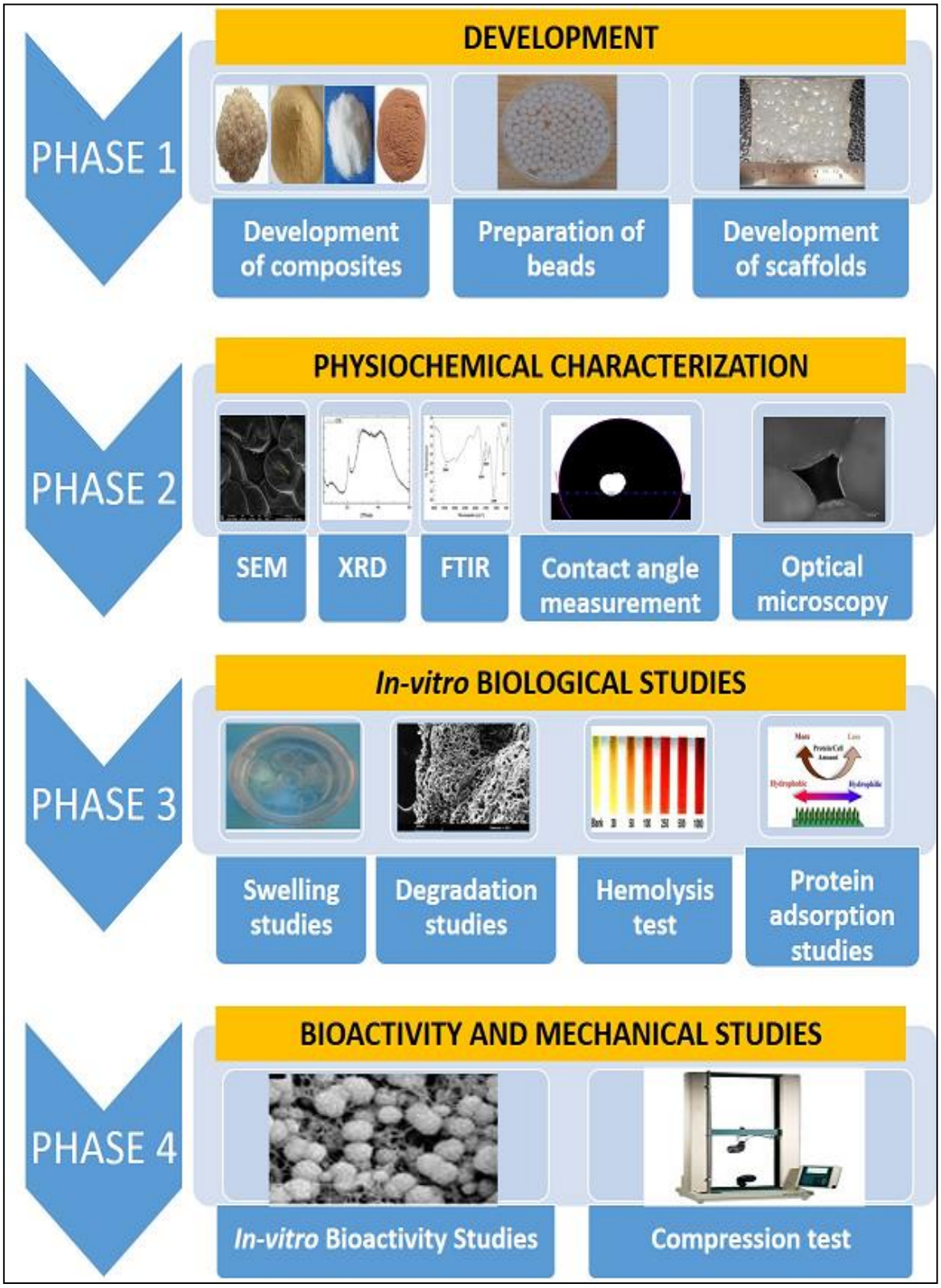
Figure 1.8. Graphical illustration of macrosphere fusing due to sintering

1.5. Objectives

In the present work a CS/MMT/HA composite was developed and further used for the production of sintered macrospheric 3D scaffolds. Further the composite scaffolds were coated with carboxymethyl tamarind seed powder (CTSP).The scaffold fabrication method is based on direct agglomeration of the macrospheres. The defined methodology allows fabrication of scaffolds which are porous and have controllable shape and pore size distribution. The fabricated samples were then subjected to sintering, which is known to improve the mechanical properties of the composite. The CS/MMT/HA composite 3D scaffolds were characterized using attenuated total reflectance-Fourier transform infrared (ATR-FTIR), X-ray diffraction (XRD), and scanning electron microscopy (SEM). The scaffolds were further assessed for their *in vitro* bioactivity, hemocompatibility, protein adsorption, water adsorption and degradation. The mechanical properties were evaluated by analysing the compressive strength.

- Solid state synthesis of hydroxyapatite
- To develop polymer composite (Chitosan /Montmorillonite /Hydroxyapatite/ Tamarind seed powder) macrospheric three dimensional scaffolds.
- To characterize the processed composite scaffolds.
- To perform *in-vitro* biological studies.
- To compare the different variants of the composite scaffolds for their biological and mechanical properties.

1.6. Work Plan


PHASE 1
DEVELOPMENT
Development of composites
Preparation of beads
Development of scaffolds
PHASE 2
PHYSIOCHEMICAL CHARACTERIZATION
SEM
XRD
FTIR
Contact angle measurement
Optical microscopy
PHASE 3
In-vitro BIOLOGICAL STUDIES
Swelling studies
Degradation studies
Hemolysis test
Protein adsorption studies
PHASE 4
BIOACTIVITY AND MECHANICAL STUDIES
In-vitro Bioactivity Studies
Compression test

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

Over the years, a huge advancement in the fields of surgical replacement and organ transplantation to treat organ or bone tissue loss. Be that as it may, the deficiencies connected with autografting and allografting transplantation, for example, inalienable contributor site restrictions, tissue dismissal and infections have led to the advancement of tissue building, which recovers new tissue utilizing cells, bioactive atoms and scaffolds. The grafts comprising polymers and ceramics materials might have beneficial properties, for example, biodegradation, biocompatibility and mechanical strength [24] .

Polymers which are biodegradable should deteriorate in the body without producing any of toxic items in the tissues. Biodegradation of the polymers can be acquired by cutting hydrolytically unsteady links in the back bone of the polymer. An insert fabricated from polymers which are biodegradable can be built to degrade at a rate that will gradually exchange burden to the mending spine. These polymers ought to have: (i) polymers and their decay items ought to be evoke any immunogenic response or any lethality; (ii) degradation and retention rates ought to be equilibrium with the regeneration of the tissue ; and (iii) polymeric materials ought to have magnificent mechanical properties similar to that of the human tissues [25].

Biocompatibility shows the capacity of materials to accomplish its role with a proper host reaction, in a particular application. This incorporates both structural as well as surface compatibility. Surface similarity implies the compound, organic, physical (counting surface morphology) appropriateness of an insert morphology to the tissue of the host. Auxiliary similarity is the ideal adjustment to the mechanical conduct of the tissues of the host. Once inserted in the body, the mechanical properties of regular bone change with their organic area on the grounds that the crystallinity, porosity and arrangement of bone acclimate to the natural and biomechanical situations [26]. The great fracture toughness as well tensile strength of bone are credited to the intense and adaptable collagen filaments fortified by hydroxyapatite (HA).

The mechanical power of scaffolds is of great significance in tissue engineering, since they are closely related to the stable structure and strength in practical applications. The scaffolds are desired to have sufficient mechanical power to work appropriately starting from the implantation time to the completion of tissue regeneration process. A number of factors can be accounted for the mechanical response of multi-component composite scaffold, like the

size of particle of the inorganic components, the intrinsic mechanical properties of the organic component, as well as the interactions among the inorganic and organic components, the ratio of the inorganic/organic content and character of cross linking [27]. Furthermore, a decline in scaffold porosity and rise in pore wall thickness, significantly increase the mechanical properties. As the scaffold relies on the pore wall to pass the stress and bear the load, the structure of the pores and the pore wall thickness will affect the mechanical properties substantially. The scaffold with lesser concentration has the thinner pore wall and higher porosity. It was observed that if the porosity was raised and pores were enlarged, it resulted in decrease of mechanical strength.

The scaffold strength is of specific significance in tissue designing, since they are firmly connected to the durability and stability of the implant. The scaffolds should have enough mechanical quality to work successfully from the time of grafting to the completion of tissue regeneration. Numerous elements can add to the mechanical reaction of multi-segment composite implant, for example, the molecule size of the inorganic segments, the characteristic mechanical properties of the implant material and the interfacial associations between the inorganic and natural polymers, the proportion of the inorganic/natural substance and cross connecting character [28]. Besides, a decrease in framework porosity and an expansion in pore wall thickness, enormously upgrade the mechanical properties. As the implant relies upon the pore wall to infuse the applied stress and bear the load, the pore structure and the thickness of pore wall will influence the mechanical properties considerably. The implant with lower concentration has the thinner pore wall and higher porosity. Increasing porosity and growing pores brought about reduction of mechanical quality [29].

Chitosan and its subsidiaries are exceptionally appealing biomaterials in the scaffold composites on account of their tendency to degrade while the new tissue is regenerated, in the end without provocative responses or dangerous degradation. Tang *et al*. demonstrated that the common Cs and HA composites have an excellent biocompatibility with the hard tissue and an incredible osteoconductivity. They likewise proposed that this composite might be appropriate for simulated bone embeds and edge materials of tissue building. Jayabalan *et al.* have reported a nano composite containing calcinated HA particles is both biocompatible and osteocompatible. They additionally demonstrated that HA could conceivably enhance both the mechanical properties and biocompatibility of bone uniting materials. The electro spun nano composite nano filaments of HA/chitosan with their

composition and structure close to the common mineralized nanofibril partners were set up by Zhaang *et al* [30]. The report stated that these nano composite filaments are of potential use for bone tissue building applications. Kaashiwazaki *et al.* have created unique CS/HA nano composites with permeable structure by the co-precipitation and porogen filtering strategy. These composites were discovered to have both enhanced biocompatibility as well as biodegradation. Chitosan nano strands with a normal measurement ranging from 100 to 50 nm were effectively arranged by means of electrospinning of chitosan and poly vinyl liquor mix arrangement. A CS/HA nano hybrid implant with high porosity and uniform nanostructure had additionally been created through a bionic treatment joined with thermally affected phase separation. These nHA particles highly affected coordinating crystallization of apatite in the framework and prompted the great bioactivity of the nano cross breed platform. Zhaao *et al*. established a CS/HA–gelatin network (HA/CS-Gel) composite which was biodegradable and had a comparative organization to that of ordinary human bone readied as a 3D biomimetic platform by stage detachment technique for bone tissue building. Zhaao et al. manufactured two sorts of biomimetic composite materials, chitosan–gelatin (CG) and hydroxyapatite/chitosan–gelatin (HCG) and showed upgraded protein and calcium particle adsorption properties of HA in the CG polymer system which enhanced introductory cell-attachment and long term development, support osteogenic separation upon incitement, and also kept up the progenicity of the 3D human mesenchymal immature microorganism (hMSCs) builds. Liuyuun *et al*. stated the physicochemical and natural properties of a unique biodegradable composite platform made of nano-hydroxyapatite and polymers of chitosan and carboxymethylated cellulose, in particular, nHAp/CS/CMC, which was set up by stop drying technique. The platform was non-lethal and had great cell biocompatibility, and the consequences of implantation investigation in vivo demonstrated that the framework has great tissue biocompatibility and has an awesome potential to be utilized as bone tissue designing material [31].

The major challenge in developing scaffolds with sufficient mechanical properties presents a unique difficulty in the field of 3D tissue engineering. The test of creating scaffolds with sufficient mechanical properties displays a one of a kind issue in tissue engineering. PCNs appear to have colossal potential concerning the part of accomplishing satisfactory mechanical properties. The work on the utilization of MMT for the arrangement of PCNs was brought to light by the Toyota research group when they stated critical change in mechanical properties of the PCNs made out of little MMT loadings. Consequently, MMT

was utilized for the arrangement of PCNs in different studies that reported increment in mechanical properties, diminish in gas diffusion and combustibility, and impact on biodegradability after the expansion of MMT [32].

Macrospheres are naturally streaming particles made out of inorganic and/or polymeric materials. Ordinarily, macrospheres are utilized as vehicles for controlled discharge since they can beat the downsides of ordinary treatment and upgrade the viability of the given medication. As of late, because of the fast advancement of tissue designing, macrospheres have been utilized as a part of tissue engineered scaffolds. In macrosphere-based/containing scaffolds, macrospheres assume an imperative part in both medication discharge and cell habitation. There are two sorts of macrospheres chiefly displaying scaffold properties, which are macrosphere sintered and macrosphere joined scaffolds. Taken together, the improvement of macrosphere-based/containing scaffolds may open another entryway for developing medication discharge platforms for bone regenerative applications [33].

The development of three dimensional (3D) scaffold based implants have become an important method for bone healing and regeneration in the field of tissue engineering. Bone scaffolds must have high porosity as well good mechanical strength. The interconnected pores are important as they are indispensable for cell ingrowth, proliferation and nutrient transport in the scaffold. The mechanical properties should be similar to that of the natural bone to prevent stress shielding. However, the scaffolds with high porosity tend to have poor mechanical strength. In recent years various methods free drying, electrospinning etc. have been explored which provide polymeric scaffolds with high porosity. These scaffolds do not possess enough mechanical strength. In order to obtain bone scaffolds with both porosity and high mechanical strength we need to optimize both suitable biomaterial and the method of fabrication of scaffold. The defined methodology allows fabrication of scaffolds which are porous and have controllable shape and distribution of pore size.

One of the earliest sintered macrosphere scaffolds (SMSs) were reported in 2001 by Borden *et al.* These SMSs not only aided in controlled release of drugs but also facilitated cell proliferation due to their porous structure. It was seen that compared to the traditional 3D scaffolds, SMS significantly enhances the mechanical properties. The compressive strength of these scaffolds was found equal to that of the cancellous bone. A graphical illustration of SMS preparation is shown in Figure. Currently there are two different ways to fuse the macrospheres together for the fabrication of the scaffolds. The first method involves heating

of the macropheres which are stacked in a mould, above the glass transition temperature (T_g) of for several hours. Thereafter the mould is removed to obtain the desired 3D scaffolds. The second method involves use of a suitable solvent to bond the macrospheres together.

Three dimensional (3-D) scaffolds are commonly used for drug delivery, investigation of cell behaviour and material studies in the field of tissue engineering. Three-dimensional scaffolds are typically porous, biocompatible and biodegradable materials that serve to provide suitable microenvironments, that is, mechanical support, physical, and biochemical stimuli for optimal cell growth and function. The porosity and pore size of 3-D scaffolds have direct effects on their use during biomedical applications. Networks which are opwn and interconnected are vital for cell nutrition, proliferation, and migration for vascularization of tissue and formation of new tissues [34]. A porous surface also serves to facilitate mechanical interconnection between the scaffolds and adjoining tissue to improve the mechanical stability of the implant. In addition, the network structure of the pores assists in guiding and promoting new tissue formation. Materials with high porosity enable effective release of biofactors such as proteins, genes, or cells and provide good substrates for nutrient exchange. However, the mechanical property that is important in maintaining the structural stability of the biomaterial is often compromised as the result of increased porosity. Hence, an equilibrium between the mass transport and mechanical function of the scaffolds should exist for an ideal scaffold system. As a result, the final porosity and pore sizes of the scaffold should be taken into account in accordance to the intended eventual application during scaffold design and fabrication stages. This review is focused on the fabrication of porous 3D scaffolds, the methods for evaluating porosity and pore sizes of 3-D constructs, and some of the reported effects of pore size and porosity on cell behaviour and overall mechanical properties. A more general overview of the various scaffold fabrication and porosity measurement techniques has been covered in previous reviews [35].

Three dimensional (3-D) scaffolds are normally utilized for medication conveyance, examination of cell conduct and material studies in the field of tissue building. 3-D scaffolds are commonly porous, biocompatible and biodegradable materials that serve to give appropriate microenvironments, that is, mechanical backing, physical, and biochemical jolts for ideal cell development and capacity. The porosity and pore size of 3-D platforms have direct effects on their usefulness amid biomedical applications. Open permeable and interconnected systems are key for cell sustenance, multiplication, and movement for vascularization of tissue and development of new tissues. A permeable surface additionally

serves to encourage mechanical interconnection between the platforms and encompassing tissue to enhance the mechanical dependability of the insert. What's more, the system structure of the pores helps with directing and advancing new tissue development [36]. Materials with high porosity empower successful arrival of biofactors, for example, proteins, qualities, or cells and give great substrates to supplement trade. Be that as it may, the mechanical property that is imperative in keeping up the auxiliary security of the biomaterial is regularly traded off as the aftereffect of expanded porosity. Consequently, a harmony between the mass transport and mechanical strength of the scaffolds ought to exist for an ideal platform framework. Thus, the last porosity and pore sizes of the framework ought to be considered in understanding to the planned possible application amid platform configuration and manufacture stages. This audit is centred around the creation of permeable 3-D scaffolds, the strategies for assessing porosity and pore sizes of 3-D builds, and a portion of the reported impacts of pore size and porosity on cell conduct and general mechanical properties. A broader outline of the different scaffold manufacture and porosity estimation methods has been secured in past surveys [37].

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Hydroxyapatite synthesis by ammoniacal precipitation

HA was synthesized by ammoniacal precipitation method, with a constant Ca/P ratio of 1.67. In the solid state synthesis of HA diammonium phosphate ($(NH_4)_2HPO_4$) and calcium nitrate tetra hydrate ($Ca(NO_3)_2.4H_2O$) were used as predecessors of phosphate and calcium, respectively. $NaHCO_3$ as a gas foaming agent. A mortar pestle was used for grounding and blending the reactants. The blended reactants were then kept for aging for about 24 h. The by-products were removed by washing the reactants a number of times with ethanol and deionized water. The washed reactants were kept in a hot air oven at 80 °C for 6 h and then calcinated for 1 h at 650 °C for obtaining HA.

3.1.2. Preparation of chitosan macrospheres

The biopolymer solution was prepared by dissolving chitosan (2 w/v %) in an aqueous solution of glacial acetic acid (2 v/v %). The polymer solution was then added drop wise through a capillary into a gently stirring coagulation solution (1 N sodium hydroxide and 26 v/v % ethanol). The coagulated macrospheres were then filtered and washed multiple times with distilled water to obtain neutrality. Similarly macrospheres were also prepared with the respective chitosan composite solutions [38]. The three variations of chitosan composite solutions prepared were: CS/HA, CS/MMT and CS/HA/MMT. The CS/MMT and CS/HA composite solutions were prepared by dissolving 10 w/w % of MMT and HA in the solution of pure CS respectively. The CS/MMT/HA composite was prepared by dissolving 10 w/w% [39]. In order to prepare the CS composite solutions the corresponding reinforcements were added to the pure biopolymer solutions and stirred overnight until a homogenous solution was obtained.

3.1.3. Preparation of uncoated chitosan composite 3D macrospheric scaffolds

The filtered and neutralized macrospheres were vacuum dried for 24 h before being used for 3-D scaffold fabrication. The vacuum dried macrospheres were then moulded with the help of a cylindrical plastic mould. The mould can be of any desired dimensions. After the macropspheres were stacked in the mould it is washed with 2 ml solution of glacial acetic acid (1 v/v %) for 60 s. The scaffold is then compressed with the back of the plunger of a

syringe. This was followed by washing with 5 % NaOH solution for 60 s. The acetic acid was causes the outer layer of the macrospheres to dissolve which leads to joining of the adjacent macrospheres under the compression of the plunger. The NaOH wash assures that the macrospheres do not dissolve completely under the effect of the acetic acid by neutralizing the acid. The scaffold is then removed from the mould and incubated in a water bath at for 24 h at 37 °C.

3.1.4. Preparation of CTSP coated chitosan composite 3-D macrospheric scaffolds

The CTSP solution was prepared by dissolving 5 w/v % CTSP in distilled water and stirring the solution overnight until a homogenous solution is obtained. The filtered and neutralized macrospheres were vacuum dried for 24 h before being used for 3-D scaffold fabrication. The vacuum dried macrospheres were then moulded with the help of a cylindrical plastic mould. The mould can be of any desired dimensions. After the macropspheres were stacked in the mould it is washed with 2 ml solution of glacial acetic acid (1 v/v %) for 60 s. The scaffold is then compressed with the back of the plunger of a syringe. This was followed by washing with 5 % NaOH solution for 60 s. The acetic acid was causes the outer layer of the macrospheres to dissolve which leads to joining of the adjacent macrospheres under the compression of the plunger. The NaOH wash assures that the macrospheres do not dissolve completely under the effect of the acetic acid by neutralizing the acid. The scaffolds were then dipped in the CTSP solution for 24 h.

3.1.5. Sintering of coated and uncoated chitosan composite 3D scaffolds

The final step of chitosan composite 3-D scaffold fabrication comprises of sintering. The samples were kept on glass slide and covered with a glass beaker to create a controlled environment for sintering. The said set up also insures that there is not loss of moisture during the sintering process. The scaffolds were sintered at 140 °C, which is the glass transition temperature of chitosan for 1 h. The sintering process increase. The sintering process is known to enhance the surface bonding of the macrospheres and also increase the mechanical strength of chitosan. The sintered chitosan composite scaffolds were then stored in a vacuum desiccator for future use. The entire process of scaffold preparation is shown in Figure 3.1. The composition of various chitosan composite scaffolds along with their respective codes is given in Table 3.1.

Table 3.1. Composition of CS composite scaffolds with their respective codes

Serial No.	Sample Composition	Sample Code
1.	Uncoated Chitosan (2 wt/v %)	CS
2.	Uncoated Chitosan/Montmorillonite (2 wt/v % and 10 wt/wt %)	CSM
3.	Uncoated Chitosan/Hydroxyappatite (2 wt/v% and 10 wt/wt %)	CSH
4.	Uncoated Chitosan/Montmorillonite/Hydroxyappatite (2 wt/v % and 10 wt/wt %)	CSHM
5.	CTSP Coated Chitosan (2 wt/v %)	CS’
6.	CTSP Coated Chitosan/Montmorillonite (2 wt/v% and 10 wt/wt %)	CSM’
7.	CTSP Coated Chitosan/Hydroxyappatite (2 wt/v% and 10 wt/wt %)	CSH’
8.	CTSP Uncoated Chitosan/Montmorillonite/Hydroxyappatite (2 wt/v % and 10 wt/wt %)	CSHM’

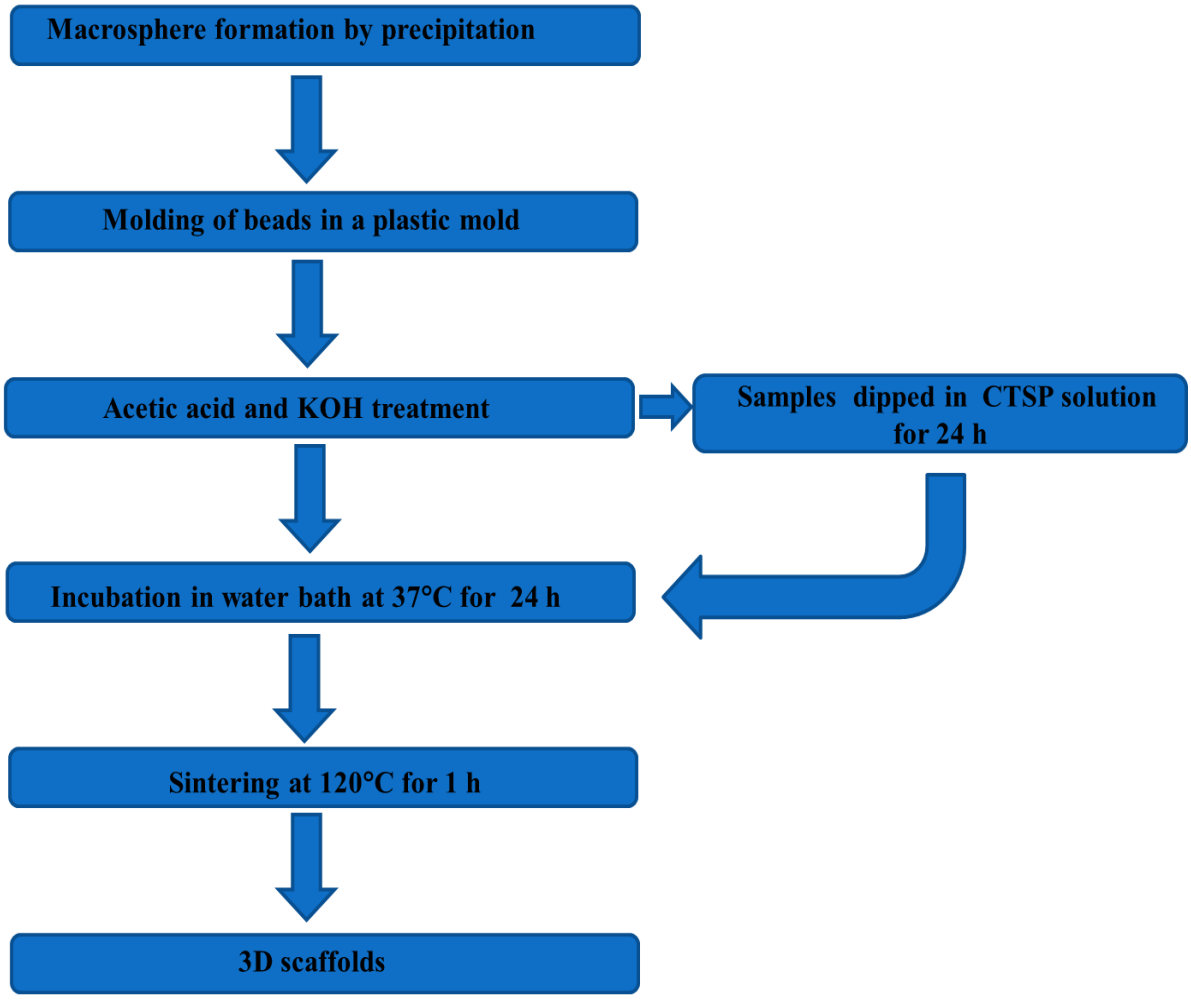


Figure 3.1. Methodology of macrospheric scaffold fabrication

3.1.6. X-ray diffraction analysis (XRD)

The XRD graphs of chitosan composite scaffolds (CS, CSM, CSH, CSMH, CS', CSM', CSH' and CSHM') were obtained to investigate phase content of composite scaffolds for a scanning range of 2θ =5-60º. The readings were taken at a scanning rate of 5º /min having step size of 0.05º, using Rigaku Ultima IV diffractometer (Japan) with Cu Kα radiation (λ=1.5418 Å), operating at 40 kV.

3.1.7. Scanning electron microscopy (SEM)

The morphology of the surface and size of pores of the prepared chitosan composite were examined using the SEM (JOEL JSM 6480LV, USA). Prior to analysis, samples were cut into very thin slices with a razor blade and coated with gold using a sputter coater and then samples were analysed at 15 kV.

3.1.8. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR spectra of the chitosan composite scaffolds was attained using the Bruker, Alpha E spectrophotometer (Germany). The transmittance spectra was obtained in the range of 4000–520 cm^{-1} at a spectral resolution of 4 cm^{-1}. The ATR-FTIR spectra gives a clear picture about the molecular interaction between the individual constituents of the chitosan composite.

3.1.9. Protein adsorption study

The adsorption of protein on the surface of the composite scaffolds was studied by using BSA as the standard protein solution and Bradford assay to quantify the amount of protein adsorbed. The BSA solution was prepared in phosphate buffer saline (PBS). The samples were cut in equal sizes (1 cm × 1 cm) and dipped in BSA (1 mg/ml BSA in PBS) solution for 24 h at 37 °C. After were then centrifuged at 4000 rpm for 10 minutes. The quantification of the protein adsorbed was done by a 100 µl aliquot of the non-adsorbed protein solution mixed with 1 ml of Bradford reagent and 2 ml of distilled water. The concentration of adsorbed protein was analysed by UV spectrophotometer measurement at 595 nm using a previously calibrated plot. In order to accurately determine variations in the concentration of adsorbed protein on the different composite scaffolds the readings were taken in triplicates for each sample.

3.1.10. Hemocompatibility studies

In order to determine whether the chitosan composite scaffolds are compatible with the microenvironment of the body it is essential to perform the hemocompatibility test. Prior to the test the samples were immersed in 10 ml of physiological saline for 24 h. The test was performed with goat blood mixed with trisodium citrate and diluted upto 0.8 v/v % with physiological saline. The samples were immersed in 0.5 ml of the diluted goat blood for 1 h at 37 °C. After incubation the samples were centrifuged at 6000 rpm for 10 minutes. The physiological saline was used as the negative control whereas a solution of 0.5 ml of blood added into 10 ml of 0.01N HCl was used as the positive control. The absorbance (OD) was recorded at 545 nm [40]. The hemolysis percentage (%) was calculated using the following equation

$$\% \text{ Haemolysis} = \left(\frac{OD_{test} - OD_{-ve}}{OD_{+ve} - OD_{-ve}}\right) X100 \quad (1)$$

3.1.11. Water absorption study

The swelling capacity of the chitosan composite scaffolds was determined in phosphate buffer saline (PBS). The scaffolds were cut into pieces of equal size (1 cm × 1 cm) and their dry weight was measured. The scaffolds were then immersed in 10 ml PBS at room temperature. The scaffolds were taken out at an interval of 1 h, the surface was dried and the samples were weighed to determine the wet weight of the samples. The procedure was repeated till the scaffolds showed almost constant weight [41]. The swelling percentage (S_w %) was calculated using the following equation

$$S_w(\%) = \left[\frac{W_w - W_d}{W_w}\right] X\ 100\% \quad (2)$$

where W_w is the wet weight of the sample whereas the W_d is the dry original weight of the sample.

3.1.12. Degradation studies

The *in vitro* degradation of chitosan composite scaffolds was evaluated by soaking the samples in PBS solution and the degradation percentage was calculated for 2, 4 and 6 weeks respectively. The scaffolds were cut into pieces of equal sizes (1 cm × 1 cm) and weighed. The samples were then soaked in 10 ml PBS and maintained at a constant temperature of 37

°C. The PBS solution was not replaced during the experiment so that the change in pH at the end of the second, fourth and sixth week can be recorded. At the end of the respective weeks the scaffolds were taken out and the pH of the PBS was measured. The scaffolds were washed with water and dried at 40 °C for 4 days. After drying the final weight of the scaffolds was recorded [42]. The degradation percentage (D_w) was calculated using the following equation where W_i and W_f are the initial and final weights of the chitosan composite scaffolds respectively.

$$D_w = \left[\frac{W_i - W_f}{W_i}\right] X\ 100\% \qquad (3)$$

3.1.13. *In vitro* bioactivity studies

The chitosan composite scaffolds were analysed for the apatite formation on the surface of the scaffolds after soaking them in simulating body fluid (SBF) for 3 weeks. The SBF was prepared according to the protocol proposed by Kokubo and Takadama. The scaffolds were cut into pieces of equal sizes and soaked in SBF solution at a constant temperature of 37 °C. At the end of 3 weeks the scaffolds were then taken out, rinsed with distilled water and dried for 12 h at 37 °C. The scaffolds were then observed under SEM to evaluate the morphology of apatite formed on the chitosan composite scaffolds [43].

3.1.14. Mechanical studies

The mechanical properties of the chitosan composite scaffolds were evaluated by determining their compressive strength and Youngs modulus. The compressive strength was evaluated using the Tinius Olsen H10KS "Universal Testing Machine" as per ASTM D6641/D6641M-14 The scaffolds were fabricated with dimensions in the ration 2:1 (height: diameter). A load cell of 100 N was used with a constant rate of 1 mm/s and the scaffolds were compressed till 70 % deformation.

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. Scanning electron microscopy

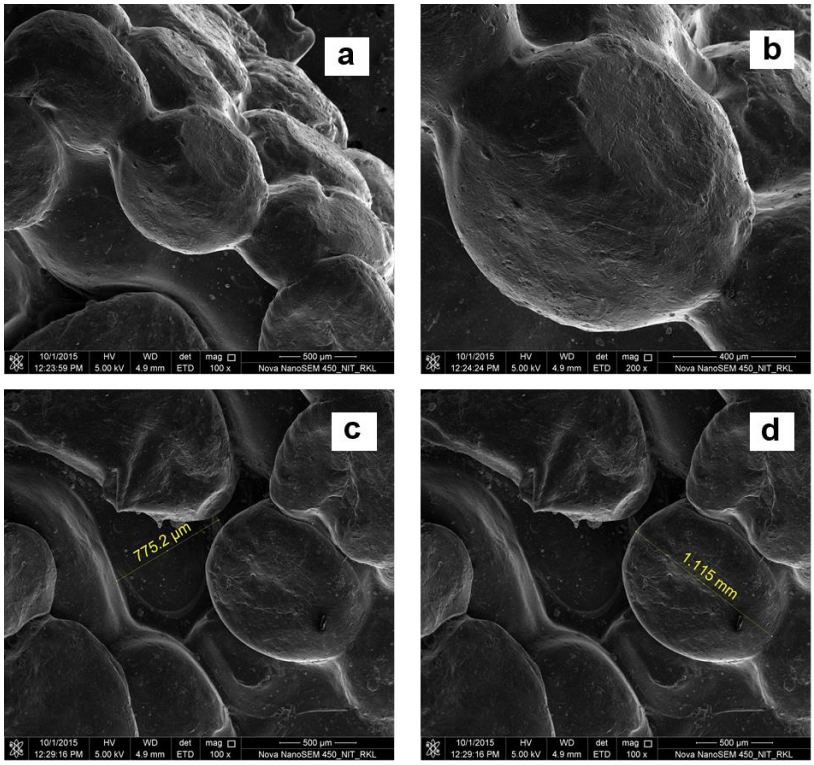


Figure 4.1. SEM micrographs of CS scaffolds

The present method of scaffold fabrication ensures an open pore structure of the scaffolds. The shape of the fabricated scaffolds and diameter of the macrospheres can be altered by varying the shape of the mould and diameter of the syringe respectively. In this study same size of syringe is used for the fabrication of the various chitosan composite macrospheres hence they have same size which is approximately 1 mm. Figure 4.1 shows the SEM micrographs of the chitosan composite scaffolds. In Figure 4.1 (a) a strong union between the adjacent macrospheres was observed. This strong union was obtained by the acetic acid treatment which caused the surface to dissolve partially. Figure 4.1 (b) shows a single macrosphere with rough surface. The roughness is caused due to vacuum drying the scaffold which lead to loss of considerable amount of water from individual chitosan composite macrosphere. It was observed that the effect of drying was seen mostly on the pure chitosan macrospheres. The CSHM composites showed least shrinkage. This can be attributed to the water retaining capacity of MMT. It was further observed that the CTSP coating also controlled the extent of shrinkage in the chitosan composite macrosphereic scaffolds. Thus the CSHM' composite scaffold minimum shrinkage among all the variants and retained the

spherical morphology even after being subjected to vacuum drying. This is a very important attribute as this will ensure that the scaffolds retain the desired shape and size. The average pore size of the scaffolds was found to be around 700 µm as shown in Figure 4.1 (c). The size of an individual macrosphere is shown in Figure 4.1 (d) which is around 1 mm. Thus the SEM micrographs confirm the open pore structure of the chitosan composite scaffolds and gives an insight into the pore size, macrophere size and surface roughness of the various chitosan composite scaffolds.

4.2. X-ray diffraction analysis

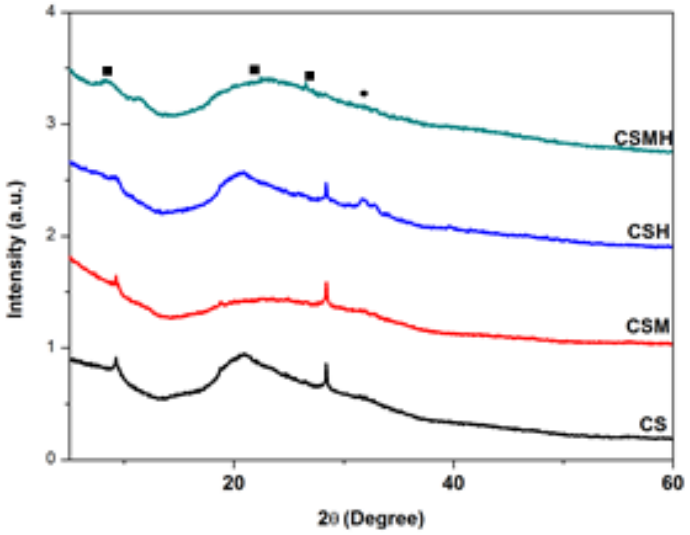


Figure 4.2. XRD pattern of uncoated CS/HA/MMT composite scaffold

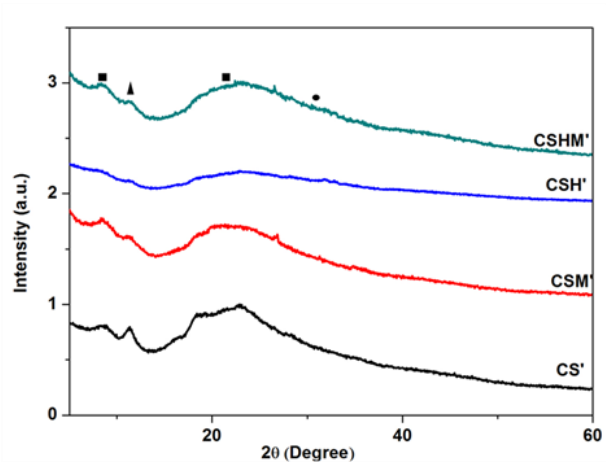


Figure 4.3. XRD pattern of CTSP coated CS/MMT/HA composite scaffolds

The XRD patterns were analysed to evaluate the structural phases in the composite scaffolds. The XRD peaks of the chitosan composite scaffolds are reported in Figure 4.2 and Figure 4.3. The principal peaks of HA appeared at values of 25.9º and 32.1º. Previous literatures have reported that in pure chitosan, there are two characteristics peaks, one at 2θ=20.02º and another one at 2θ=10.53º. In pure chitosan as well as the CS composite scaffolds, the diffraction peak at 2θ=20.02º not only gets shifted to 22.8º but also merges with other peaks, thereby creating an amorphous peak. Thus, it can be concluded that CS is present in amorphous form. This amorphous form can be attributed to the acetic acid and chitosan interaction. This interaction acts as a hindrance in inter- and intramolecular hydrogen bond formation in CS, leading to a loosely packed structure. The peak for pure MMT is found at 2θ=6.9º. The characteristics peaks of the individual components are present in the CS composite scaffolds as well. The characteristic peak of CTSP is known to be found at 2θ= 20°. This peak is merged with the amorphous peak of CS.

4.3. Attenuated total reflectance-Fourier transform infrared spectroscopy

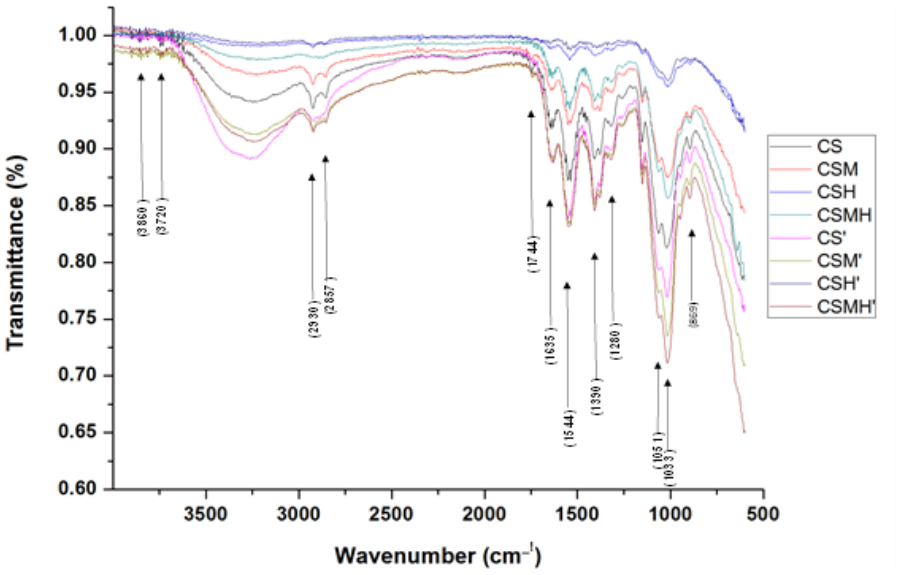


Figure 4.4. ATR-FTIR pattern of CS composite scaffolds

Attenuated Total Reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) offers definite information about chemical bonds and molecular structures of the individual components present in the composite. The ATR-FTIR spectra of the CS composite scaffolds (CS, CSM, CSMH, CS', CSM', CSH' and CSMH') are shown in Figure 4.4. In the ATR-FTIR spectrum of MMT, the bands at 527 and 1031 cm^{-1} were assigned to Si–O–Al deformation and Si–O stretching, respectively. The low intense peak consistent to OH

bending of H_2O was observed at 1639 cm^{-1}. The band related to structural OH stretching was observed at 3624 cm^{-1}. Moreover, the bands related with OH bending of water and structural OH stretching was shifted to 1655 cm^{-1} and 3626 cm^{-1}, respectively. The ATR-FTIR spectra of HA showed the peaks associated to stretching modes of PO_4^{3-} were detected at 1043 and 962 cm^{-1}. The bands between 602–567 cm^{-1} were assigned to bending vibrations of phosphate groups present in HA. The bands at 3570 and 630 cm^{-1} were related to the stretching and liberation modes of OH, respectively. Also, low intense peak of adsorbed water (stretching) was present at 1642 cm^{-1}.

4.4. Protein adsorption studies

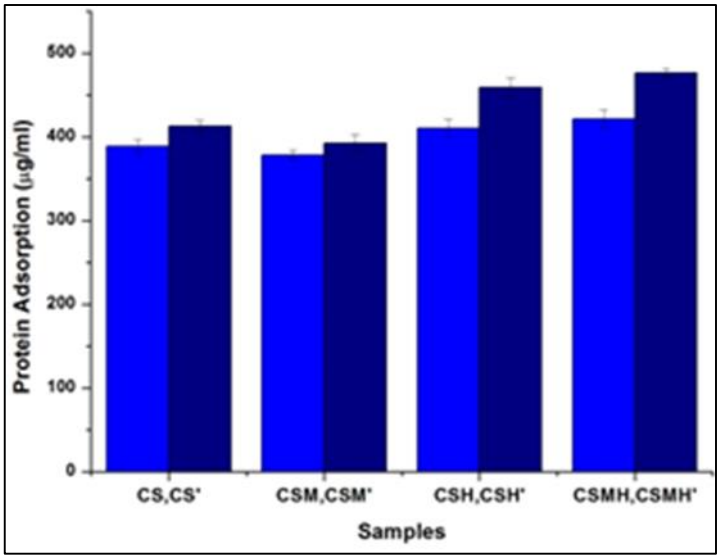


Figure 4.5. Protein adsorption graph of CS composite scaffolds

When a scaffold is implanted in the body it comes in contact with the micro environment of the body. The different proteins present in the physiological fluids are one of the major components that not only come in contact but also get adsorbed on the surface of the scaffolds. These adsorbed proteins mediate various biological responses and further help in attachment, growth and proliferation of cells on the scaffold surface. The adsorption of proteins is majorly influenced by the surface characteristics like charge, roughness, wettability and most importantly the chemistry of the surface. BSA is a glycoprotein which is structurally and functionally similar to that of human serum albumin and thus it was used to evaluate protein adsorption on the CS composite scaffolds. The protein adsorption studies helped us to understand the effect of different reinforcements on protein adsorption ability of the CS composite scaffolds. Current studies have showed that the presence of functional groups (oxygen, nitrogen, carboxyl, and hydroxyl) on the surface influence protein

adsorption. The results showed an increase in protein adsorption in the order CSM<CS<CSH<CSMH. The electrostatic force and the hydrophobic interaction are important to induce greater protein–surface affinity. The protein adsorption is shown in Figure 4.5. It is also seen that the coated variations of the same composites showed higher protein adsorption. This can be credited to the surface charge present in the CTSP molecules. Among the CTSP coated samples the protein adsorption was found in the order CSM'<CS'<CSH'<CSMH'.

4.5. Hemocompatibility studies

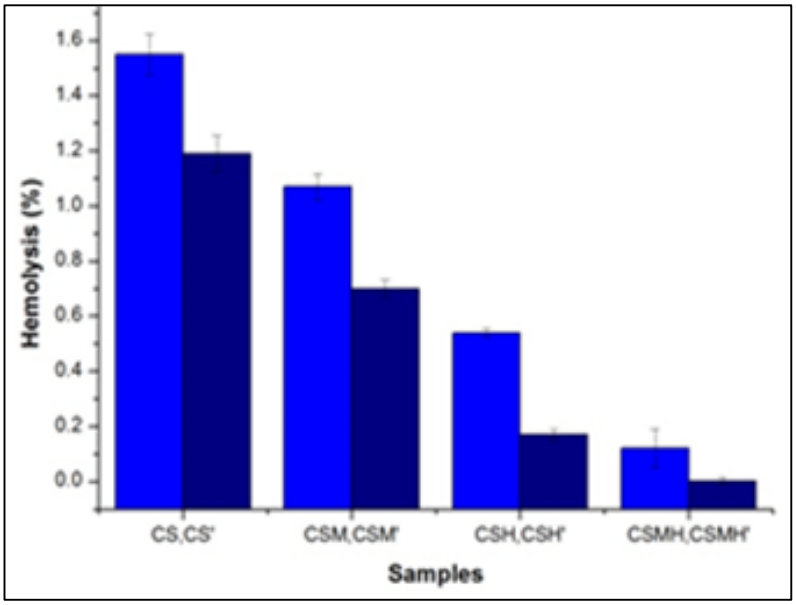


Figure 4.6. Percentage hemolysis of CS composite scaffold

The interactions between the numerous cellular components and the scaffolds inside the host body may lead to cell damage. The interactions can cause rupture of the erythrocytes which can be fatal for the body. Thus the *in vitro* interaction between the scaffold and blood is evaluated to predict the hemcompatibility of the scaffold *in vivo.* The positive and negative control showed 100 % and 0 % hemolysis respectively. The evaluation of the uncoated samples showed that the maximum hemolysis was found in pure CS scaffolds which was around 1.6 %. The minimum hemolysis was found in CSHM composite which was around 0.1 %.Thus it is seen that the addition of reinforcements like MMT and HA has reduced the haemolysis percentage thereby improving the biocompatibility of the samples. This can be due to the hydrophobic nature of both the ceramics used Further when the CTSP coated variants of the respective composite scaffolds were evaluated it was seen that the coating had significantly reduced the haemolytic nature of the scaffolds. The minimum hemolysis

was found in the CSHM' sample. It must be noted that even though there were variations in the haemolytic nature of the various composite scaffolds all the samples are considered hemocompatible as all the variants show > 5 % hemolysis. The haemolysis percentage profile of the composites is given in Figure 4.6.

4.6. Swelling studies

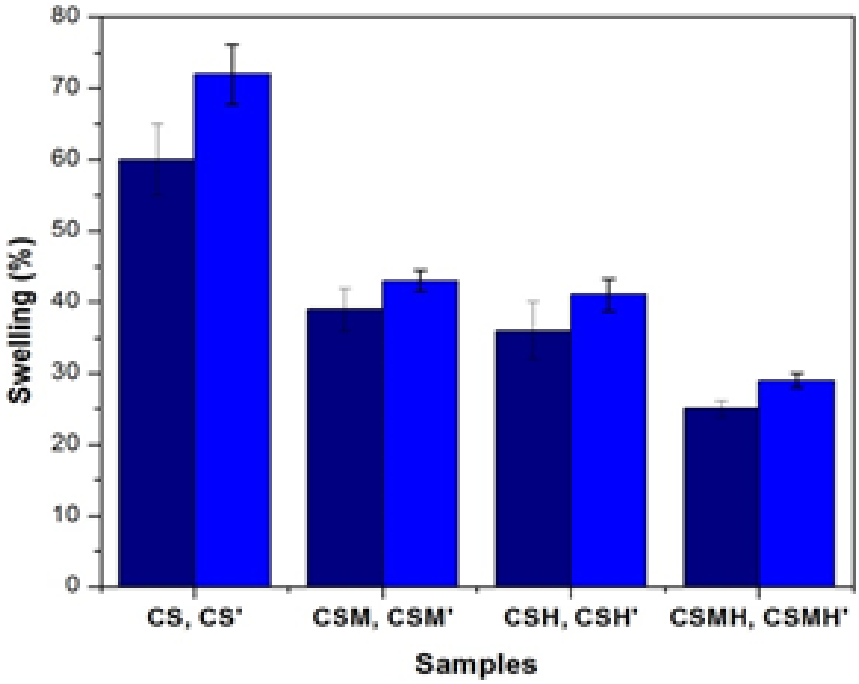


Figure 4.7. Swelling percentage of various CS composite scaffolds

When the scaffolds come in contact with the various physiological fluids of the body they have tendency to swell. This swelling tendency can affect the shape and mechanical stability of the scaffolds over the extended period of time. The swelling of scaffolds facilitates the cells in filtration into the scaffolds in a three dimensional fashion, during cell culture by increasing the pore size. However if there is an uncontrolled swelling it can reduce the mechanical strength thereby rendering it incompatible for bone tissue scaffolds. CS is known for its very high percentage of swelling which is one of the major drawbacks of the polymer especially in case of load bearing scaffolds. Thus the swelling profile of the different composite scaffolds was compared to analyse the effect of the various reinforcements on the swelling behaviour of the scaffolds. The swelling percentage profile of the various uncoated samples showed the effect of sintering on the CS composite scaffolds. It was seen that the sintering of the scaffolds at the glass transition temperature led to decrease in the swelling percentage. Further the reinforcement of CS with MMT and HA also decreased the swelling

percentage. The coating of the CS composite scaffolds with CTSP however increased the swelling percentage of the sintered composite scaffolds. The maximum swelling was seen in the CS' scaffold and the least was seen in the CSHM scaffold. . According to the results, addition of HA and MMT can affect the swelling of the CS composites. The addition of HA has induced a reduction in the swelling of the polymer matrix due to formation of a momentary HA barricade preventing water infusing into the scaffold. The addition of HA reduces the hydrophilicity of the chitosan by joining to the hydrophilic –COOH and $–NH_2$. Reinforcement of MMT also played a role in reduction of swelling. The nanosized sheets of MMT have formed a block that prevents the interaction between polymer and water molecules, leading into reduction of water content in both CSM and CSMH composite scaffolds. Therefore, the swelling behaviour of CS based composite scaffolds can be tailored by using proper amount of inorganic phase. Further it was seen that the coating of the composite samples with CTSP has increased the swelling of the polymer matrix. This is due to hydrophilic CTSP molecule coats the entire surface of the composite scaffolds. Figure 4.7 shows the swelling percentage of the various CS composite scaffolds.

4.7. Degradation studies

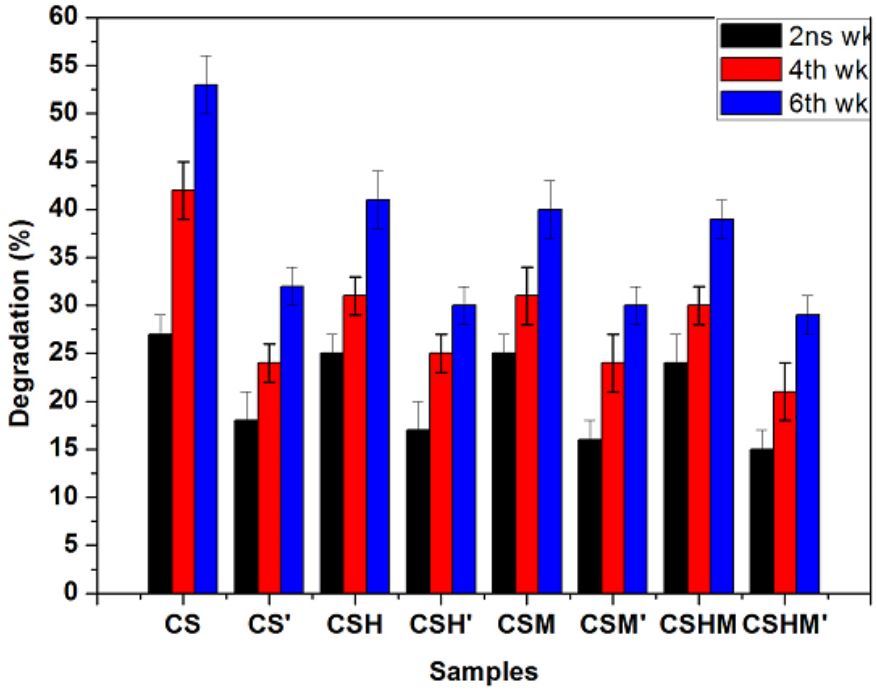


Figure 4.8. Degradation percentage of CS composite scaffolds

While engineering a new scaffold for bone tissue implantation the degradation rate of the scaffold plays a very important role. For bone tissue regeneration stable scaffolds which can

form equilibrium with the regeneration of bones are required. Chitosan is known for its very high rate of biodegradation. Although the degradation products are not harmful to the body as the polymer is a naturally occurring one but the high rate of degradation has adverse effects on the stability and longevity of the scaffold. Thus the *in vitro* biodegradation of the chitosan composite scaffolds was studied to analyse the effect of the reinforcements on the degradation rate of the scaffolds. It was observed that the CS/HA/MMT scaffold which was coated with CTSP showed the least rate of biodegradation. The reinforcement of HA and MMT individually also affected the biodegradation rate. Figure 4.8 shows the degradation profile of the various CS scaffolds for two, four and six weeks.

4.8. *In- vitro* bioactivity studies

The ability of the scaffold to bond directly with the living bone by forming bone like apatite on its surface is known as bioactivity of the implant. In the present work the bioactivity of the CS composite scaffolds was evaluated by immersing them for 21 days in SBF. After that the samples were dried and observed under SEM for evaluating the apatite formation. It is known that pure CS has very poor bioactive properties. CS lacks active sites on its surface which act as trigger for apatite formation. The addition of reinforcements especially HA enhance the bioactivity of the composite. This is due to the presence of chelating agents in the form of Ca^{2+} and PO_4^{3-} which acts as active sites. MMT and CTSP are also known to increase the bioactivity of the composite. In Figure 4.9 and 4.10 the SEM micrographs showing the apatite formation on the various uncoated and CTSP coated CS composites is shown. It is seen that hardly any apatite formation on the pure CS scaffolds. The CTSP coated sample however shows considerable amount of apatite formation when compared to pure CS scaffolds. In the other variants we have found high amount of cylindrical apatite formation. The CTSP coated scaffolds were found to be more bioactive than their respective uncoated variants. The highest bioactivity was found in the CS/HA/MMT CTSP coated sample. Thus it can be concluded that the reinforcement of HA and MMT as well as the CTSP coating has increased the bioactive nature of the composite scaffolds to a great extent.

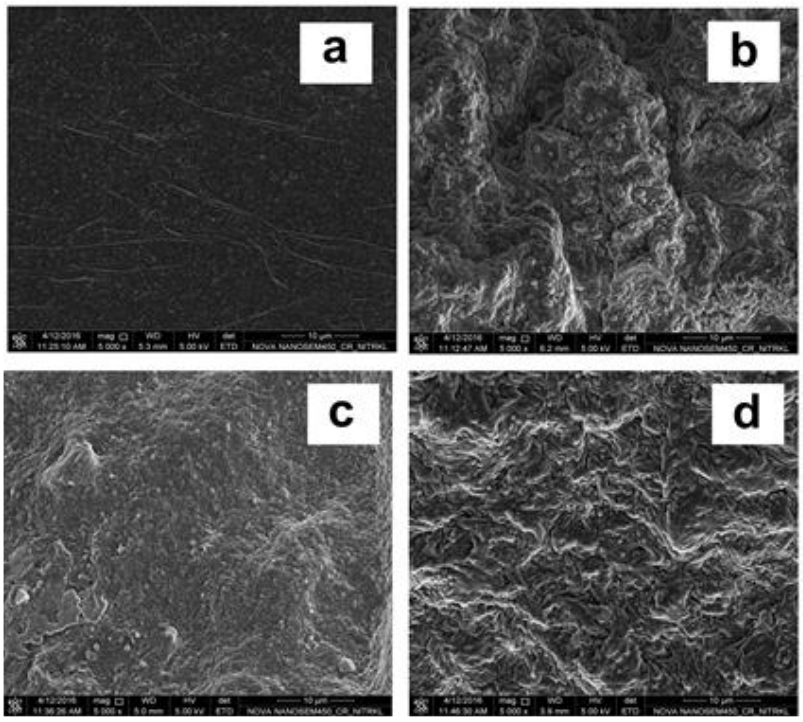


Figure 4.9. SEM micrographs showing apatite formation in (a) CS (b) CSH (c) CSH (d) CSHM

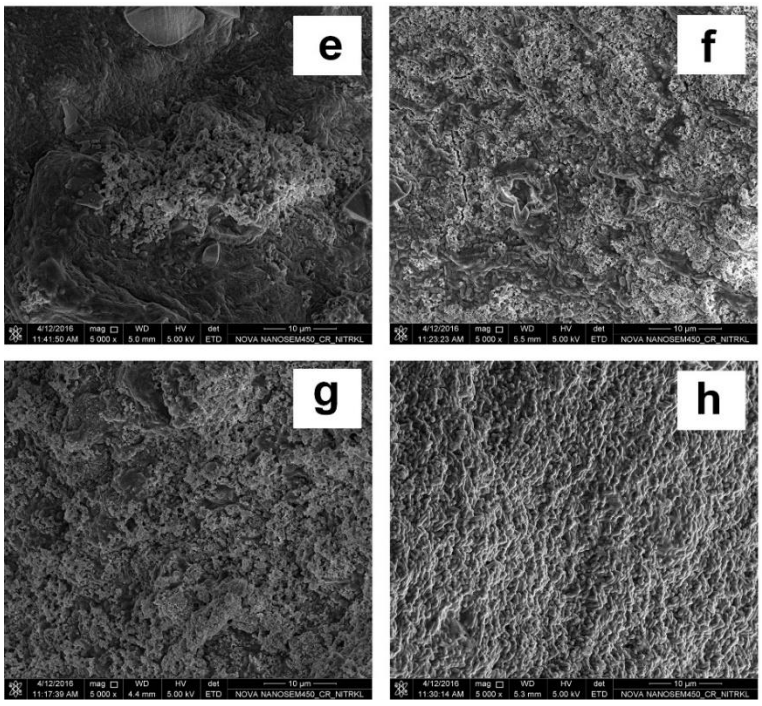


Figure 4.10. SEM micrographs showing apatite formation on CTSP coated (a) CS (b) CSH (c) CSM (d) CSHM composites

4.9. Mechanical studies

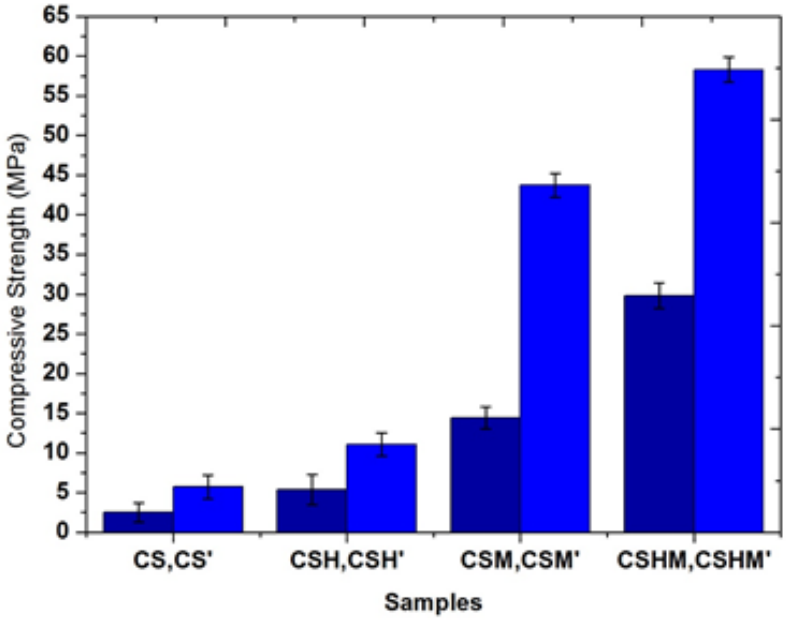


Figure 4.11. Compressive strength of CS composite scaffolds

The scaffolds must have enough mechanical strength to resist stress incurred by newly formed in vitro till its implantation *in vivo*. The main aim of this study was to determine the increase in compressive strength of the composite scaffolds. There are various factors which play an important role in affecting the strength of the scaffolds. The composites of natural polymers and ceramics is known to possess more strength than the individual constituents. Similarly the addition of nano clay can also alter the compressive strength of the scaffolds. Further the sintering of the polymer at its glass transition temperature is known to alter the mechanical properties of the polymer. Thus the mechanical strength of the various composite scaffold was evaluated to assess the effect of the various factors. It was detected that there was substantial rise in the compressive strength of the CTSP coated samples as compared to their corresponding uncoated samples. Thus it can be seen that the coating has significantly increased the compressive strength of the samples. The compressive is found to increase in the order CS<CSH<CSM<CSHM. This order is found for both coated as well as uncoated samples. The mechanical strengths of the various CS composite scaffolds is given in Figure 4.11.

5. CONCLUSION

In the present study novel macrosphere-based CS/MMT/HA composite scaffolds coated with carboxymethyl tamarind seed powder have been fabricated which have enhanced mechanical properties for load bearing bone tissue engineering applications. X-ray diffraction, scanning electron microscopy, Fourier transform infrared spectroscopy and Optical Microscopy were performed to characterize the scaffolds. Among *in vitro* studies protein adsorption, haemolysis and water absorption studies were performed was performed. The X-ray diffraction studies showed the various characteristic peaks whereas the SEM studies showed surface morphology, pore size and bead size of the composite scaffolds. The various functional groups corresponding to the individual components like CS, MMT, HA and CTSP were identified through ATR-FTIR. Protein adsorption studies showed an increase in protein adsorption in the CSM<CS<CSH<CSMH and further increase in adsorption of protein in their CTSP coated variants. The hemocompatibilty test showed that although all the samples were sufficiently hemocompatible the CTSP coating further enhanced the hemocompatible nature of the composites. The water absorption property of the composites was also enhanced by the coating. The work concludes CSMH' scaffolds fabricated with well controllable and predictable internal architecture and geometry has potential application in bone tissue engineering.

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